ASSISTANT COMMISSIONER FOR PATENTS
Washington, D.C. 20231

Case Docket No. P107158-00904
Date March 24, 2000

Sir:

Transmitted herewith for filing under 37 C.F.R. §1.53(b) is the patent application of:
Inventor(s): Akira KAWAKAMI; Fumihiro TERAMI

For: LOW TEMPERATURE EXPRESSION CHITINASE cDNAs AND METHOD FOR ISOLATING THE SAME

XX Specification (21 pages)

XX 2 sheets of drawings

XX Declaration and Power of Attorney

XX Return Receipt Postcard

XX Notification of Change of Name and Address

XX An Assignment of the invention to Hokkaido National Agricultural Experiment Station with PTO-1595

XX A certified copy of Japanese application(s) No.(s) 11-081694; dated March 25, 1999

XX A filing fee, calculated as shown below:

	(Col. 1)	(Col. 2)
FOR:	No. Filed	No. Extra
BASIC FEE		
TOTAL CLAIMS	11 - 20 =	* 0
INDEP CLAIMS	4 - 3 =	* 1
MULTIPLE DEPENDENT CLAIM PRESENTED		

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Respectfully submitted,

ARENT FOX KINTNER PLOTKIN & KAHN, PLLC

By: Douglas H. Goldhush
Reg. No. 33,125

Arent Fox Kintner Plotkin & Kahn, PLLC
1050 Connecticut Avenue, N.W., Suite 600
Washington, D.C. 20036-5339
Telephone No. (202) 857-6000
Facsimile No. (202) 638-4810
DHG/hk
Enclosures: Check #288132/Specification and Claims/Declaration/Priority Document (1)
Drawings (2 sheets)/Assignment/PTO-1595 Form/Return Receipt Postcard

TITLE OF THE INVENTION

Low Temperature Expression Chitinase cDNAs and Method for Isolating the Same

BACKGROUND OF THE INVENTION

The present invention relates to chitinase cDNAs and to a method for their isolation, and more specifically it relates to chitinase cDNAs having a function of conferring plant disease resistance under low temperature, and to a method of isolating the chitinase cDNAs.

In the northern regions, overwintering crops such as barley, forage grasses and wheat must survive subzero temperature (0 ℃ or below 0 ℃) and a long-lasting snow cover condition (0 ℃ in darkness). However, overwintering crops in such environment are often attacked by snow molds which are a diverse group of psychrophilic parasitic fungi. This biotic stress greatly limits yields and quality of biennial or perennial crops, in the same manner as a low temperature stress will do in the northern region with snow accumulation.

In current winter wheat cultivation, it is necessary to apply a broad-spectrum fungicides before a continuous snow cover for protecting the plant from snow molds infection.

However, it has taken high cost and it has been proved difficult to apply the fungicide at the effective time, because of unstable nature of the start of a snow cover every year.

In view of the above, it has been desired to raise a plant variety having a high disease resistance under low temperature environment.

Nevertheless, up till now, when using several conventional breeding methods each based on cross-breeding, it has not been possible to raise superior varieties with high resistance, and a long time (many years) is required for raising superior varieties. For this reason, there has been a strong demand for variety improvement by more effective methods such as gene engineering methods.

As a result of repeated diligent research over years aimed at solving the problems described above, the inventors of the present invention have arrived at the following conclusion. Specifically, it has been found that plant disease resistance under low temperature environment is induced by cold acclimation that occurs under a low temperature from autumn through winter (hereunder referred to as "hardening") and that expression of the three chitinase cDNAs of the invention described hereunder are found during this hardening, with the translation product conferring plant disease resistance through digestion of chitin, one of the major components of fungus cell wall.

SUMMARY OF THE INVENTION

It is therefore an object of the present invention to provide chitinase cDNAs that encode proteins having enzymatic

function in low temperature environments and that when introduced into plants confer plant disease resistance.

It is another object of the invention to provide a method for isolation of chitinase cDNAs that encode proteins having enzymatic function in low temperature environments and that when introduced into plants confer plant disease resistance.

According to one aspect of the present invention, there is provided a winter wheat-derived chitinase cDNA, characterized in that said cDNA has a nucleotide sequence corresponding to an amino acid sequence listed as SEQ. ID. No. 1 in Fig. 1. In detail, said cDNA comprises 771 nucleotides/256 amino acids and has 98% identity (on amino acid sequence level) with barley-derived chitinase cDNA. In more detail, said cDNA encodes a protein with chitinase activity in low temperature environment and confers plant disease resistance by digestion of chitin, one of the major components of fungus cell wall.

According to another aspect of the present invention, there is provided another winter wheat-derived chitinase cDNA, characterized in that said cDNA has a nucleotide sequence corresponding to an amino acid sequence listed as SEQ. ID. No. 2 in Fig. 2. In detail, said cDNA comprises 972 nucleotides/323 amino acids and has 68% identity (on amino acid sequence level) with rye-derived chitinase cDNA. In more detail, said cDNA encodes a protein with chitinase activity in low temperature environment and confers plant

disease resistance by digestion of chitin, one of the major components of fungus cell wall.

According to a further aspect of the present invention, there is provided a further winter wheat-derived chitinase cDNA, characterized in that said cDNA has a nucleotide sequence corresponding to an amino acid sequence listed as SEQ.ID. No.3 in Fig. 3. In detail, said cDNA comprises 960 nucleotides/319 amino acids and has 95% identity (on amino acid sequence level) with spring wheat-derived chitinase cDNA. In more detail, said cDNA encodes a protein with chitinase activity in low temperature environment and confers plant disease resistance by digestion of chitin, one of the major components of fungus cell wall.

According to a still further aspect of the present invention, there is provided a method of isolating a winter wheat-derived chitinase cDNA having a nucleotide sequence corresponding to an amino acid sequence listed as SEQ.ID. No.1 in Fig. 1, a winter wheat-derived chitinase cDNA having a nucleotide sequence corresponding to an amino acid sequence listed as SEQ.ID. No.2 in Fig. 2, a winter wheat-derived chitinase cDNA having a nucleotide sequence corresponding to an amino acid sequence listed as SEQ.ID. No.3 in Fig. 3, said method comprising the steps of: extracting mRNA from winter wheat variety PI173438 (having high snow molds resistance) that has undergone a sufficient hardening process; preparing cDNA and a cDNA library based on said mRNA; analyzing

nucleotide sequences of a number of plant-derived chitinase cDNAs which have all been published by EMBL/Genebank/DDBJDNA Databank; designing a pair of chitinase cDNA-specific degenerated primers with reference to highly conserved nucleotide sequence portions of the plant-derived chitinase cDNAs; conducting PCR (polymerase chain reaction) using a pair of chitinase cDNA-specific degenerated primers and using said cDNA as a template, thereby amplifying fragments of chitinase cDNAs and obtaining amplified DNA fragments; and using said amplified DNA fragments as probes for screening said cDNA library by a hybridization assay, to isolate recombinant plaques containing full length of cDNA.

In particular, one of the pair of chitinase cDNA-specific degenerated primers has the following nucleotide sequence:

(Forward): 5' C-A-C-G-A-G-A-C-C-A-C-N-G-G-C-G-G-N-T-G-G-G-C (SEQ.ID. No.4),

and the other has the following nucleotide sequence:

(Reverse): 5' A-C-N-A-A-T-A-T-C-A-T-C-A-A-C-G-G-C-G-G (SEQ.ID. No.5).

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 shows an amino acid sequence of SEQ.ID No.1.

Fig. 2 shows an amino acid sequence of SEQ.ID No.2.

Fig. 3 shows an amino acid sequence of SEQ.ID No.3.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The cDNAs of the present invention are chitinase cDNAs capable of expressing under a low temperature condition.

The method for isolating the cDNAs of the present invention may be carried out in the following manner.

Specifically, mRNA is extracted from winter wheat PI173438 (having high snow molds resistance) that has undergone a hardening process (low temperature acclimation) under natural conditions in Sapporo City, Japan until November 22. This mRNA is then used to prepare cDNA and a cDNA library.

Next, nucleotide sequences of a number of plant-derived chitinase cDNAs which have all been published by EMBL/Genebank/DDBJDNA Databank are closely analyzed, and a pair of chitinase cDNA-specific degenerated primers are designed with reference to highly conserved nucleotide sequence portions.

The pair of designed chitinase cDNA-specific degenerated primers are used in a PCR (polymerase chain reaction) using the above-mentioned cDNA as the template for amplifying the expected chitinase cDNA fragments (all are approximately 400 bp), and the amplified fragments are isolated.

The amplified fragments are used as probes for screening the cDNA library by a hybridization assay, to isolate recombinant plaques containing full length of cDNA. The nucleotide sequences of the isolated plaques were analyzed and demonstrated to be three different chitinase cDNAs which are

three kinds of chitinase cDNA fragments, all are novel in plants.

An example of the method for isolating the cDNAs of the present invention was carried out in the following steps 1) - 3).

1) Preparation of cDNA and cDNA library from snow molds resistant winter wheat variety PI173438

mRNA was extracted by a common method from the crown portion of winter wheat (Triticum astivum L.) PI173438 (having high snow molds resistance) that had been seeded in a container in late September and had then undergone a hardening process under natural conditions until November 22. A portion (5 μg) of the obtained mRNA was used to synthesize cDNA utilizing a cDNA Synthesis Kit (STRATAGENE Co.). After attaching adaptors to both ends of the cDNA, it was incorporated into a ZAP Expression Vector (STRATAGENE Co.), thereby obtaining a cDNA library of approximately 6 x 10^6 pfu.

2) PCR using a pair of cDNA-specific degenerated primers and using the cDNA as a template

One of the pair of chitinase cDNA-specific degenerated primers, having the following nucleotide sequence:

(Forward): 5' C-A-C-G-A-G-A-C-C-A-C-N-G-G-C-G-G-N-T-G-G-G-C (SEQ.ID. No.4),

the other chitinase cDNA-specific degenerated primer, having the following nucleotide sequence:

(Reverse): 5' A-C-N-A-A-T-A-T-C-A-T-C-A-A-C-G-G-C-G-G (SEQ.ID. No.5).

which were synthesized based on highly conserved regions of the nucleotide sequences of known chitinase cDNAs (published by EMBL/Genebank/DDBJDNA Databank), were used in a PCR using the cDNA (synthesized in the manner described in the above) as the template.

The PCR was performed in a final volume of 50 μl. In detail, 1 μl of Taq DNA polymerase (5 units/μl) by Nippon Gene Co., 5 μl of 10 x PCR buffer (containing $MgCl_2$), 5 μl of dNTP solution (10 mM), 2 μl of each primer (12 μM) and about 10 ng of the cDNA synthesized in the above, were mixed and then brought to a total of 50 μl with distilled water. The PCR conditions and number of reaction cycles are shown in Table 1 below.

Table 1

PCR condition and number of reaction cycles

Initial Denaturation	94° C	1 min	once
Denaturation Annealing Primer Extension	94° C 48° C 72° C	1 min 1 min 1 min	30 cycles
Final Extension	72° C	2 min	once

(In Table 1, "denaturation" refers to a reaction in which double-stranded DNA is melt into single strand and secondary structure is eliminated, "primer extension" refers to an synthesizing of the new complementary strand, and "30 cycles" means that three basic steps of denaturation-annealing-primer extension are repeated with 30 cycles.

As a result, DNA fragments (having expected length of approximately 400 bp) of chitinase cDNAs were amplified by the above PCR with the pair of chitinase cDNA-specific degenerated primer having nucleotide sequence of SEQ.ID No.4 and the primer with the nucleotide sequence of SEQ.ID No.5. Theses amplified DNA fragments were then isolated and subsequently sequenced using a DNA sequencer (Model 373S by ABI Co.) according to the conventional method. By comparing the

sequences with known chitinase, it were confirmed that novel chitinase cDNA fragments (having a high homology with known chitinase cDNA) were isolated.

3) Isolation and nucleotide sequencing of full length cDNAs encoding chitinase of the present invention

About $1x10^5$ recombinant plaques from the cDNA library obtained in the manner described in the above were subjected to a hybridization assay by using filters lifted with $1x10^5$ recombinant plaques, and using probes prepared by labeling (with ^{32}P) each novel chitinase cDNA fragment obtained in the above.

The hybridization reaction was carried out for 16 hours at 42° C, in a solution containing 50% formamide, 5 x SSPE, 5 x Denhardt's solution, 0.5% SDS and 0.2 mg/ml salmon sperm DNA with ^{32}P-labeled probe.

The filters were then washed twice in a solution containing 2 x SSC and 0.1% SDS at 65 ℃ for 10 min. Afterwards, the filters were washed twice with another washing solution containing 0.1 x SSC and 0.1% SDS, at 65 ℃ for 15 min. Detection of each positive plaque binding to ^{32}P-labed probe was performed by exposing above washed filters to X-ray films.

About 45 positive recombinant plaques obtained in the above were subjected to nucleotide sequencing with DNA sequencer by ABI Co.

Analysis of the nucleotide sequences of these recombinant plaques revealed that novel chitinase cDNAs having nucleotide sequences corresponding to the amino acid sequences listed as SEQ.ID Nos. 1 - 3 in Figs. 1 - 3 had been isolated from winter wheat variety PI173438.

In fact, what were isolated were i) a novel winter wheat-derived chitinase cDNA having a nucleotide sequence corresponding to the amino acid sequence listed as SEQ.ID. No.1 in Fig. 1, comprising 771 nucleotides/256 amino acids and having 98% identity (on amino acid sequence level) with barley-derived chitinase cDNA, ii) a novel winter wheat-derived chitinase cDNA having a nucleotide sequence corresponding to the amino acid sequence listed as SEQ.ID. No.2 in Fig. 2, comprising 972 nucleotides/323 amino acids and having 68% identity (on amino acid sequence level) with rye-derived chitinase cDNA, iii) a novel winter wheat-derived chitinase cDNA having a nucleotide sequence corresponding to the amino acid sequence listed as SEQ.ID. No. 3 in Fig. 3, comprising 960 nucleotides/319 amino acids and having 95% identity (on amino acid sequence level) with spring wheat-derived chitinase cDNA.

Investigation of Enzymatic Activity

In order to investigate enzymatic activities of the novel chitinase cDNAs of the present invention, enzymatic reactions were conducted under the following conditions using

culture solutions containing novel proteins secreted by recombinant yeast (into which each novel chitinase cDNA of the present invention has been introduced).

[Enzymatic Reaction Condition]

Buffer solution (20 mM citric acid/phosphoric acid), pH 4.5

Final substrate concentration: 1% collidal chitin

Reaction temperature: 38 ℃, reaction time: 16 hours.

As a result, it was confirmed that the culture solutions containing novel proteins secreted by recombinant yeast (into which each novel chitinase cDNA of the present invention has been introduced) had a chitinase activity capable of producing a disaccharide (a chito-oligosaccharide) or a trisaccharide (another chito-oligosaccharide) from chitin polymer (serving as a substrate).

The nucleotide sequences of the novel cDNAs obtained in the present invention are listed in the following.

Nucleotide Sequence of cDNA corresponding to the Amino Acid Sequence Listed as SEQ.ID. No.1

10	20	30	40	50	60
ATGGCGAGGT	TTGCTGCCCT	CGCCGTGTGC	GCCGCCGCGC	TCCTGCTCGC	CGTGGCGGCG
70	80	90	100	110	120
GGGGGTGCCG	CGGCGCAGGG	CGTGGGCTCG	GTCATCACGC	GGTCGGTGTA	CGCGAGCATG
130	140	150	160	170	180
CTGCCCAACC	GCGACAACTC	GCTGTGCCCG	GCCAGAGGGT	TCTACACGTA	CGACGCCTTC
190	200	210	220	230	240
ATCGCCGCCG	CCAACACCTT	CCCGGGCTTC	GGCACCACCG	GCAGCGCCGA	CGACATCAAG
250	260	270	280	290	300
CGCGACCTCG	CCGCCTTCTT	CGGCCAGACC	TCCCACGAGA	CCACCGGAGG	GACGAGAGGC
310	320	330	340	350	360
GCTGCCGACC	AGTTCCAGTG	GGGCTACTGC	TTCAAGGAAG	AGATAAGCAA	GGCCACGTCC
370	380	390	400	410	420
CCACCATACT	ATGGACGGGG	ACCCATCCAA	TTGACAGGGC	GGTCCAACTA	CGATCTTGCC
430	440	450	460	470	480
GGGAGAGCGA	TCGGGAAGGA	CCTGGTGAGC	AACCCAGACC	TAGTGTCCAC	GGACGCGGTG
490	500	510	520	530	540
GTGTCCTTCA	GGACGGCCAT	GTGGTTCTGG	ATGACGGCGC	AGGGAAACAA	GCCGTCGTGC
550	560	570	580	590	600
CACAACGTCG	CCCTACGCCG	CTGGACGCCG	ACGGCCGCCG	ACACCGCTGC	CGGCAGGGTA
610	620	630	640	650	660
CCCGGATACG	GAGTGATCAC	CAATATCATC	AACGGCGGGC	TCGAGTGCGG	AATGGGCCGG
670	680	690	700	710	720
AACGACGCCA	ACGTCGACCG	CATCGGCTAC	TACACGCGCT	ACTGCGGCAT	GCTCGGCACG
730	740	750	760	770	780
GCCACCGGAG	GCAACCTCGA	CTGCTACACC	CAGAGGAACT	TCGCTAGCTA	G.........

Nucleotide Sequence of cDNA corresponding to the

Amino Acid Sequence Listed as SEQ. ID. No. 2

```
        10          20          30          40          50          60
ATGTCCACGC  TGAGAGCGCG  GTGTGCGACG  GCCGTCCTGG  CCGTCGTCCT  GGCGGCCGCC

        70          80          90         100         110         120
GCGGTCACGC  CGGCCACGGC  CGAGCAGTGC  GGCTCGCAAG  CCGGCGGCGC  CAAGTGCGCC

       130         140         150         160         170         180
GACTGCCTGT  GCTGCAGCCA  GTTCGGGTTC  TGCGGCACCA  CCTCCGACTA  CTGCGGCCCC

       190         200         210         220         230         240
CGCTGCCAGA  GCCAGTGCAC  TGGCTGCGGT  GGCGGCGGCG  GCGGGGTGGC  CTCCATCGTG

       250         260         270         280         290         300
TCCAGGGACC  TCTTCGAGCG  GTTCCTGCTC  CATCGCAACG  ACGCAGCGTG  CCTGGCCCGC

       310         320         330         340         350         360
GGGTTCTACA  CGTACGACGC  CTTCTTGGCC  GCCGCCGGCG  CGTTCCCGGC  CTTCGGCACC

       370         380         390         400         410         420
ACCGGAGACC  TGGACACGCG  GAAGCGGGAG  GTGGCGGCCT  TCTTCGGCCA  GACCTCTCAC

       430         440         450         460         470         480
GAGACCACCG  GCGGGTGGCC  CACCGCGCCC  GACGGCCCCT  TCTCATGGGG  CTACTGCTTC

       490         500         510         520         530         540
AAGCAGGAGC  AGGGCTCGCC  GCCGAGCTAC  TGCGACCAGA  GCGCCGACTG  GCCGTGCGCA

       550         560         570         580         590         600
CCCGGCAAGC  AGTACTATGG  CCGCGGCCCC  ATCCAGCTCA  CCCACAACTA  CAACTACGGA

       610         620         630         640         650         660
CCGGCGGGCC  GCGCAATCGG  GGTGGACCTG  CTGAACAATC  CGGACCTGGT  GGCCACGGAC

       670         680         690         700         710         720
CCGACAGTGG  CGTTCAAGAC  GGCGATATGG  TTCTGGATGA  CGACGCAGTC  CAACAAGCCG

       730         740         750         760         770         780
TCGTGCCATG  ACGTGATCAC  GGGGCTGTGG  ACTCCGACGG  CCAGGGATAG  CGCAGCCGGA

       790         800         810         820         830         840
CGGGTACCCG  GGTATGGTGT  CATCACCAAC  GTCATCAACG  GCGGGATCGA  ATGCGGCATG

       850         860         870         880         890         900
GGGCAGAACG  ACAAGGTGGC  GGATCGGATC  GGGTTCTACA  AGCGCTATTG  TGACATTTTC

       910         920         930         940         950         960
GGCATCGGCT  ACGGGAATAA  CCTCGACTGC  TACAACCAAT  TGTCGTTCAA  CGTTGGGCTC

       970         980         990        1000        1010        1020
GCGGCACAGT  GA.......  ..........  ..........  ..........  ..........
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Nucleotide Sequence of cDNA Corresponding to the

Amino Acid Sequence Listed as SEQ.ID. No.3

```
        10         20         30         40         50         60
ATGAGAGGAG TTGTGGTGGT GGGCATGCTG GCCGCGGCCT TCGCCGTGTC TGCGCACGCC

        70         80         90        100        110        120
GAGCAATGCG GCTCGCAGGC CGGCGGGGGC ACGTGCCCCA ACTGCCTCTG CTGCAGCAAG

       130        140        150        160        170        180
TTCGGTTTCT GCGGCACCAC CTCCGACTAC TGCGGCACCG GCTGCCAGAG CCAGTGCAAT

       190        200        210        220        230        240
GGCTGCAGCG GCGGCACCCC GGTACCGGTA CCGACCCCCT CCGGCGGCGG CGTCTCCTCC

       250        260        270        280        290        300
ATTATCTCGC AGTCGCTCTT CGACCAGATG CTGCTGCACC GCAACGACGC GGCGTGCCTG

       310        320        330        340        350        360
GCCAAGGGGT TCTACAACTA CGGCGCCTTC GTCGCCGCGG CCAACTCGTT CTCGGGCTTC

       370        380        390        400        410        420
GCGACCACAG GTAGCACCGA CGTCAAGAAG CGCGAGGTGG CCGCGTTCCT CGCTCAGACT

       430        440        450        460        470        480
TCCCACGAGA CGACCGGCGG GTGGCCGACG GCGCCCGACG GCCCCTACTC CTGGGGCTAC

       490        500        510        520        530        540
TGCTTCAACC AGGAGCGCGG CGCCACCTCC GACTACTGCA CGCCGAGCTC GCAGTGGCCA

       550        560        570        580        590        600
TGTGCGCCGG GCAAGAAGTA CTTCGGGCGC GGGCCCATCC AGATCTCACA CAACTACAAC

       610        620        630        640        650        660
TACGGGCCGG CGGGGCAGGC CATCGGCACC GACCTGCTCA ACAACCCGGA CCTTGTGGCG

       670        680        690        700        710        720
TCGGACGCGA CCGTGTCGTT TAAGACGGCG TTGTGGTTCT GGATGACGCC GCAATCACCC

       730        740        750        760        770        780
AAGCCTTCGA GCCACGACGT GATCACGGGC CGGTGGAGCC CCTCGGGCGC CGACCAGGCG

       790        800        810        820        830        840
GCGGGGAGGG TGCCTGGGTA CGGTGTGATC ACCAACATCA TCAACGGTGG GCTCGAGTGC

       850        860        870        880        890        900
GGGCGCGGGC AGGACGGCCG TGTCGCCGAC CGGATCGGGT TCTACAAGCG CTACTGCGAC

       910        920        930        940        950        960
CTCCTTGGCG TCAGCTACGG TGACAACCTG GACTGCTACA ACCAAAGGCC GTTCGCATAG

       970        980        990       1000       1010       1020
.......... .......... .......... .......... .......... ..........
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The advantages of the present invention may be concluded as follows.

According to the present invention there are provided novel chitinase cDNAs in wheat that have different amino acid sequences from known chitinase cDNAs and confer high disease resistance in low temperature environment. Because the three chitinase cDNAs of the present invention are capable of digesting chitin at low temperature, the introduction of any one of these three different chitinase cDNAs into plants can confer plant disease resistance in low temperature environments, so that plant varieties can be provided with high resistance against psychrophilic plant pathogens such as snow molds.

While the presently preferred embodiments of the this invention have been shown and described above, it is to be understood that these disclosures are for the purpose of illustration and that various changes and modifications may be made without departing from the scope of the invention as set forth in the appended claims.

WHAT IS CLAIMED IS:

1. A winter wheat-derived chitinase cDNA, characterized in that said cDNA has a nucleotide sequence corresponding to an amino acid sequence listed as SEQ.ID. No.1 in Fig. 1.

2. A winter wheat-derived chitinase cDNA according to claim 1, characterized in that said cDNA comprises 771 nucleotides/256 amino acids and has 98% identity (on amino acid sequence level) with barley-derived chitinase cDNA.

3. A winter wheat-derived chitinase cDNA according to claim 1, characterized in that said cDNA encodes a protein with chitinase activity in low temperature environment and confers plant disease resistance by digestion of chitin, one of the major components of fungus cell wall.

4. A winter wheat-derived chitinase cDNA, characterized in that said cDNA has a nucleotide sequence corresponding to an amino acid sequence listed as SEQ.ID. No.2 in Fig. 2.

5. A winter wheat-derived chitinase cDNA according to claim 4, characterized in that said cDNA comprises 972 nucleotides/323 amino acids and has 68% identity (on amino acid sequence level) with rye-derived chitinase cDNA.

6. A winter wheat-derived chitinase cDNA according to claim 4, characterized in that said cDNA encodes a protein with chitinase activity in low temperature environment and confers plant disease resistance by digestion of chitin, one of the major components of fungus cell wall.

7. A winter wheat-derived chitinase cDNA, characterized in that said cDNA has a nucleotide sequence corresponding to an amino acid sequence listed as SEQ. ID. No. 3 in Fig. 3.

8. A winter wheat-derived chitinase cDNA according to claim 7, characterized in that said cDNA comprises 960 nucleotides/319 amino acids and has 95% identity (on amino acid sequence level) with spring wheat-derived chitinase cDNA.

9. A winter wheat-derived chitinase cDNA according to claim 7, characterized in that said cDNA encodes a protein with chitinase activity in low temperature environment and confers plant disease resistance by digestion of chitin, one of the major components of fungus cell wall.

10. A method of isolating a winter wheat-derived chitinase cDNA having a nucleotide sequence corresponding to an amino acid sequence listed as SEQ. ID. No. 1 in Fig. 1, a winter wheat-derived chitinase cDNA having a nucleotide sequence corresponding to an amino acid sequence listed as SEQ. ID. No. 2

in Fig. 2, a winter wheat-derived chitinase cDNA having a nucleotide sequence corresponding to an amino acid sequence listed as SEQ.ID. No.3 in Fig. 3, said method comprising the steps of:

extracting mRNA from winter wheat variety P1173438 (having high snow molds resistance) that has undergone a sufficient hardening process;

preparing cDNA and a cDNA library based on said mRNA;

analyzing nucleotide sequences of a number of plant-derived chitinase cDNAs which have all been published by EMBL/Genebank/DDBJDNA Databank;

designing a pair of chitinase cDNA-specific degenerated primers with reference to highly conserved nucleotide sequence portions of the plant-derived chitinase cDNAs;

conducting PCR (polymerase chain reaction) using a pair of chitinase cDNA-specific degenerated primers and using said cDNA as a template, thereby amplifying fragments of chitinase cDNAs and obtaining amplified DNA fragments; and

using said amplified DNA fragments as probes for screening said cDNA library by a hybridization assay, to isolate recombinant plaques containing full length cDNA.

11. The method according to claim 10, wherein one of said a pair of chitinase cDNA-specific degenerated primers has the following nucleotide sequence:

(Forward): 5' C-A-C-G-A-G-A-C-C-A-C-N-G-G-C-G-G-N-T-G-G-C

(SEQ.ID. No.4),

and the other has the following nucleotide sequence:

(Reverse): 5' A-C-N-A-A-T-A-T-C-A-T-C-A-A-C-G-G-C-G-G

(SEQ.ID. No.5).

Low Temperature Expression Chitinase cDNAs

and Method for Isolating the Same

Abstract of the Disclosure

A winter wheat-derived chitinase cDNA is provided which has a nucleotide sequence corresponding to an amino acid sequence listed as SEQ.ID. No.1 in Fig. 1. Another winter wheat-derived chitinase cDNA is provided which has a nucleotide sequence corresponding to an amino acid sequence listed as SEQ.ID. No.2 in Fig. 2. Further, a winter wheat-derived chitinase cDNA is provided which has a nucleotide sequence corresponding to an amino acid sequence listed as SEQ.ID. No.3 in Fig. 3. Moreover, a method is provided for isolating the above three kinds of chitinase cDNAs.

FIG.1

AMINO ACID SEQUENCE OF SEQ. ID No1.

```
        10         20         30         40         50         60
MARFAALAVC AAALLLAVAA GGAAAQGVGS VITRSVYASM LPNRDNSLCP ARGFYTYDAF

        70         80         90        100        110        120
IAAANTFPGF GTTGSADDIK RDLAAFFGQT SHETTGGTRG AADQFQWGYC FKEEISKATS

       130        140        150        160        170        180
PPYYGRGPIQ LTGRSNYDLA GRAIGKDLVS NPDLVSTDAV VSFRTAMWFW MTAQGNKPSC

       190        200        210        220        230        240
HNVALRRWTP TAADTAAGRV PGYGVITNII NGGLECGMGR NDANVDRIGY YTRYCGMLGT

       250        260        270        280        290        300
ATGGNLDCYT QRNFAS*... .......... .......... .......... ..........
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FIG.2

AMINO ACID SEQUENCE OF SEQ. ID No2.

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        10         20         30         40         50         60
MSTLRARCAT AVLAVVLAAA AVTPATAEQC GSQAGGAKCA DCLCCSQFGF CGTTSDYCGP

        70         80         90        100        110        120
RCQSQCTGCG GGGGGVASIV SRDLFERFLL HRNDAACLAR GFYTYDAFLA AAGAFPAFGT

       130        140        150        160        170        180
TGDLDTRKRE VAAFFGQTSH ETTGGWPTAP DGPFSWGYCF KQEQGSPPSY CDQSADWPCA

       190        200        210        220        230        240
PGKQYYGRGP IQLTHNYNYG PAGRAIGVDL LNNPDLVATD PTVAFKTAIW FWMTTQSNKP

       250        260        270        280        290        300
SCHDVITGLW TPTARDSAAG RVPGYGVITN VINGGIECGM GQNDKVADRI GFYKRYCDIF

       310        320        330        340        350        360
GIGYGNNLDC YNQLSFNVGL AAQ*...... .......... .......... ..........
```

FIG.3

AMINO ACID SEQUENCE OF SEQ. ID No3.

```
        10         20         30         40         50         60
MRGVVVVAML AAAFAVSAHA EQCGSQAGGA TCPNCLCCSK FGFCGTTSDY CGTGCQSQCN

        70         80         90        100        110        120
GCSGGTPVPV PTPSGGGVSS IISQSLFDQM LLHRNDAACL AKGFYNYGAF VAAANSFSGF

       130        140        150        160        170        180
ATTGSTDVKK REVAAFLAQT SHETTGGMPT APDGPYSWGY CFNQERGATS DYCTPSSQMP

       190        200        210        220        230        240
CAPGKKYFGR GPIQISHNYN YGPAGQAIGT DLLNNPDLVA SDATVSFKTA LWFWMTPQSP

       250        260        270        280        290        300
KPSSHDVITG RWSPSGADQA AGRVPGYGVI TNIINGGLEC GRGQDGRVAD RIGFYKRYCD

       310        320        330        340        350        360
LLGVSYGDNL DCYNQRPFA* .......... .......... .......... ..........
```

N, M, M & O Docket No. _____ NIKAIDO, MARMELSTEIN, MURRAY & ORAM LLP

Declaration For U.S. Patent Application

As a below named inventor, I hereby declare that:
My residence, post office address and citizenship are as stated below my name.
I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled
(Insert Title) "Low Temperature Expression Chitinase cDNAs and Method for Isolating the Same"
the specification of which is attached hereto unless the following box is checked:

☐ was filed on _____ as United States Application Number or PCT International Application Number _____ and was amended on _____ (if applicable).

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claim(s), as amended by any amendment referred to above.
I acknowledge the duty to disclose information which is material to patentability as defined in 37 C.F.R. §1.56.
I hereby claim foreign priority benefits under 35 U.S.C. §119(a)-(d) or §365(b) of any foreign application(s) for patent or inventor's certificate, or §365(a) of any PCT International application which designated at least one country other than the United States, listed below and have also identified below any foreign application for patent or inventor's certificate or PCT International Application having a filing date before that of the application(s) for which priority is claimed:

				Priority Claimed
(List prior foreign applications. See note A on back of this page)	11-81694 (Number)	Japan (Country)	25/03/1999 (Day/Month/Year Filed)	☒ Yes ☐ No
	(Number)	(Country)	(Day/Month/Year Filed)	☐ Yes ☐ No
	(Number)	(Country)	(Day/Month/Year Filed)	☐ Yes ☐ No

I hereby claim the benefit under 35 U.S.C. §119(e) of any United States provisional application(s) listed below.

(Application Number) (Filing Date)

(Application Number) (Filing Date)

(See Note B on back of this page) ☐ See attached list for additional prior foreign or provisional applications.

I hereby claim the benefit under 35 U.S.C. §120 of any United States application(s) or §365(c) of any PCT International application(s) designating the United States of America listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior application(s) (U.S. or PCT) in the manner provided by the first paragraph of 35, U.S.C. §112, I acknowledge the duty to disclose information which is material to patentability as defined in 37 C.F.R. §1.56 which became available between the filing date of the prior application and the national or PCT International filing date of this application.

(List prior U.S. Applications or PCT International applications designating the U.S.)	(Application Serial No.)	(Filing Date)	(Status) (patented, pending, abandoned)
	(Application Serial No.)	(Filing Date)	(Status) (patented, pending, abandoned)

And I hereby appoint as principal attorneys David T. Nikaido, Reg. No. 22,663; Charles M. Marmelstein, Reg. No. 25,895; George E. Oram, Jr., Reg. No. 27,931; Robert B. Murray, Reg. No. 22,980; Martin S. Postman, Reg. No. 18,570; E. Marcie Emas, Reg. No. 32,131; Douglas H. Goldhush, Reg. No. 33,125; Kevin C. Brown, Reg. No. 32,402; Monica Chin Kitts, Reg. No. 36,105; and Richard J. Berman, Reg. No. 39,107.

Please direct all communications to the following address: NIKAIDO, MARMELSTEIN, MURRAY & ORAM LLP
Metropolitan Square
655 Fifteenth Street, N.W., Suite 330 - G Street Lobby
Washington, D.C. 20005-5701
(202) 638-5000 Fax: (202) 638-4810

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further, that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

(See Note C on back of this page)

Full name of sole or first inventor Akira KAWAKAMI
Inventor's signature Akira Kawakami March 16, 2000 (Date)
Residence Hokkaido, Japan
Citizenship Japan
Noshishukusha 1-201, 1, Hitsujigaoka, Toyohira-ku,
Sapporo-shi. Hokkaido 062-8555 Japan

Full name of second joint inventor, if any Fumihiro TERAMI

Inventor's signature Fumihiro Terami Date March 16, 2000

Residence Hokkaido, Japan

Citizenship Japan

Post Office Address Noshishukusha 1-304, 1, Hitsujigaoka, Toyohira-ku, Sapporo-shi, Hokkaido 062-8555 Japan

Full name of third joint inventor, if any

Inventor's signature Date

Residence

Citizenship

Post Office Address

Full name of fourth joint inventor, if any

Inventor's signature Date

Residence

Citizenship

Post Office Address

Full name of fifth joint inventor, if any

Inventor's signature Date

Residence

Citizenship

Post Office Address

Full name of sixth joint inventor, if any

Inventor's signature Date

Residence

Citizenship

Post Office Address

Full name of seventh joint inventor, if any

Inventor's signature Date

Residence

Citizenship

Post Office Address

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re the application of:

KAWAKAMI et al.

Serial Number: New application

Filed: March 24, 2000

For: LOW TEMPERATURE EXPRESSION CHITINASE cDNAs AND METHOD FOR ISOLATING THE SAME

NOTIFICATION OF CHANGE OF NAME AND ADDRESS

Assistant Commissioner for Patents
Washington, D.C. 20231

March 24, 2000

Sir:

Kindly change the correspondence name and address for the above-identified application to the following:

ARENT FOX KINTNER PLOTKIN & KAHN
1050 Connecticut Avenue, N.W., Suite 600
Washington, D.C. 20036-5339
Telephone: (202) 857-6000
Facsimile: (202) 638-4810

Should any fees be due with respect to this paper, please charge Counsel's Deposit Account No. 01-2300.

Respectfully submitted,

ARENT FOX KINTNER PLOTKIN & KAHN

Douglas H. Goldhush
Attorney for Applicants
Reg. No. 33,125

Atty. Docket No. P107156-90004
1050 Connecticut Avenue, N.W., Suite 600
Washington, D.C. 20036-5339
Tel: (202) 857-6000
Fax (202) 638-4810
DHG/hk